STRUCTURE AND REACTIVITY OF THE PHOSPHOTRIESTERASE ACTIVE SITE

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ABSTRACT

The structure and reactivity of the native, mutant, and metal substituted phosphotriesterase (PTE) is determined by *ab initio* quantum chemistry calculations. The x-ray structure for the Zn-Zn enzyme is leveraged into a catalytically competent active site in which a wide range of theoretical structures can be optimized for metal substituted and mutant active sites. The structural behavior of the active site is modeled using a new effective potential for representing the protein molecular environment (electrostatic, polarization, repulsive) interacting in the quantum Hamiltonian. The new methodology, effective fragment potentials (EFP), has been implemented in the GAMESS suite of electronic structure codes to make theoretical calculations on structure, spectroscopy, and reactivity tractable for systems involving many hundreds of atoms. Specific results on the structure of active site histidine to cysteine mutants, and a new proposal on the nucleophile for this hydrolase, will be presented.

INTRODUCTION

This study will focus on the theoretical analysis of the reactive behavior of organophosphorus hydrolase from *Pseudomonas diminuta*, phosphotriesterase (PTE), which catalyzes the hydrolysis of paraoxon, sarin, soman, and other inhibitors of acetylcholinesterase [1, 2]. This enzyme has been extensively studied with much emphasis on the effect on reactivity with metal substitution and mutations [3-8]. X-ray structures, of the bimetallic Zn-Zn [9] and Cd-Cd [10] enzymes, provide the basis for studying the detailed binding and mechanism. The molecular aspects of the mechanism can be deduced from the geometric and electronic structure of the reactant and transition state complexes with the enzyme active site. This information is very difficult to obtain experimentally. Structures of analogues of the reactant bound in the active site have been obtained [9, 11]. These structures provide insight into the possible binding modes. However, we suggest that not all binding modes have been catalogued even with new data showing several possibilities for simple substrates [11]. Even more important the arrangement of waters bound in the active site is poorly understood. Arrangements of the substituents in the active site are not consistent from substrate to substrate leading Benning et al to suggest “that nonproductive complexes are readily formed during the binding” and “one must proceed with caution when using x-ray structural data alone in the redesign of enzyme active sites” [11].

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The structural basis for binding is still not clear since the three regions identified as interacting with the substrate have both hydrophobic and hydrophillic residues [9, 12, 13, 14]. The mutations engineered to alter chirality are considered to work on the basis of size of the binding pocket or the steric interactions of the residues with the substrate [14]. However, the importance of two histidines (H254, H257) and tryptophan (W131) in binding suggest that hydrogen bonding interactions are also possible. For example, W131 is hydrogen-bonded to the phosphonate inhibitor in the Zn-Zn enzyme crystal structure [9] (see fig.1). Modeling of paraoxonase into the active site also shows that the nitro group would be hydrogen bonded to H257. Mutations of the residues in the first shell [15], in near active site residues towards the protein interior [7], and near active site residues toward solution [6] modify activity and specificity. The molecular basis for these changes also has not been determined although an examination of the active site of the Zn-Zn enzyme shows that a combination of steric and hydrogen-bonded interactions are probable.

Experimental observations of the binding show only two binding arrangements so far. The substrate is hydrogen bonded to second-shell residues and also interacts with dispersion interactions but phosphoryl oxygen does not bind to the metal cation with the approach to the Zn2 atom not closer than 3.4 Å. The second binding arrangement does find the phosphoryl oxygen bound to the Zn2 with a distance of 2.1 Å. However, the presence of W131 makes the binding of substrates with bulky substituents to Zn2 difficult. The data would suggest that a substituent larger than a methyl group has steric interactions that impede direct binding to Zn2.

However, the observation of binding or near binding to Zn2 encouraged experimental suggestions that the hydrolysis initiated by binding to Zn2 which

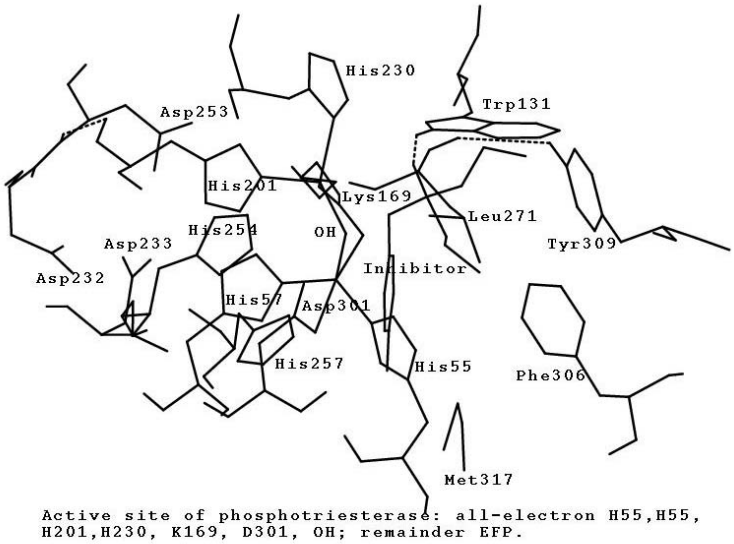


Figure1. Schematic of the active site of phosphotriesterase.

destabilized the hydroxide binding so it could act as the nucleophile [9]. However, recent structural data suggests that the binding of the phosphoryl oxygen does not destabilize hydroxide binding [11]. The theoretical calculations show that the direct binding of the phosphate to either Zn1 or Zn2 leaves the hydroxide as a bridging ligand. We suggest that waters bound to the active site will act as the nucleophile in either binding mode. Binding of water to the active site in the presence of the substrate is then part of the reactive substrate

complex but has not been studied either experimentally or theoretically.

Metal substitution is known to alter activity and specificity [3, 7] but the molecular and electronic basis is not known The accuracy with which molecular geometry must be known to provide insight into the electronic structure is also higher than is obtainable by x-ray crystal structure [16]. Differences of a few hundredths of an Å are significant in characterizing the electronic structure of the carboxylate bound to the metal in either the aspartate or carbamylate but several tenths of an Å is achieved at the resolution obtained for these enzymes. Kinetic isotope effects for the hydrolysis reaction for one substrate suggesting a late transition state have been obtained [4, 17]. However, there is evidence that for most substrates the rate is not limited by the chemical reaction [17, 18]. Theory can then contribute significantly to the understanding of the mechanism at the molecular level. We have recently developed a methodology for calculating the binding and energetic properties along the enzyme reaction path for selected catalytically competent protein structures leveraged from a single experimental structure to metal substituted or mutant structures [19-21]. Insight into the catalytic properties at the molecular level is obtained from the binding of the reactant and transition state in the active site of the enzyme. Preliminary results for phosphotriesterase already show that deductions on binding and the reaction path obtained from experiment do not completely cover the range of possible reaction paths. Accurate calculation of the Cd-Cd enzyme structure was used to validate the methodology for phosphotriesterase [20]. In this note HisXCys structures for all mutants in the active site are described. A specific reaction path in phosphotriesterase for the hydrolysis of trimethylphosphate has been calculated but the binding of trimethylphosphate and water only will be described very briefly due to the limitation on space.

METHOD

Application of *ab initio* methods is essential for accurate prediction of the properties of enzyme active sites and their role in catalysis. Although *ab initio* quantum methods have enjoyed great success in characterizing transition states and determining reaction paths *in vacuo*, the proper description of an enzyme active site can require hundreds of atoms making traditional all-electron methods intractable. An accurate picture of the binding and reactivity can be leveraged from limited experimental data with the use of effective fragment potentials (EFP) that make the calculation of the protein environment tractable [22]. The reactant, transition state, inhibitor, and product in the enzyme active site can now be optimized with a fully quantum chemical treatment of the interaction with the protein environment represented by the EFP in the hamiltonian. The EFP method provides insight into the electronic and geometric aspects of catalysis on the molecular level as well as the relative energetics along a reaction path for one of a range of catalytically competent conformations of the protein. Catalytically competent conformations are obtained from the crystal structure or from analysis of trajectories in a molecular dynamics simulation [21]. The number of waters bound in the active site or the arrangement of internal hydrogen bonds in the active site has been found to characterize different catalytic conformations for chorismate mutase. Preliminary evidence for water binding in phosphotriesterase suggests that activity in this enzyme similarly depends on water binding in the active site. Initial structures for EFP calculations are obtained from an x-ray structure, MD simulations, many-body classical mechanics [23], or semi-empirical quantum chemistry.

The effective fragment potential method was designed to model solvation effects [24-30] and is appropriately extended to describe protein interactions. The EFP are implemented in GAMESS [31] and have recently been applied to a variety of biomolecule problems [19-21, 32-37]. In the EFP method, the model of the enzyme active site is divided into two regions, an active (A) region and a spectator (S) region. The A region is treated all-electron and includes

any residues that are directly involved in the chemistry or whose movement is critical to catalysis. The S region, which influences A through electrostatic, hydrogen-bonded, and repulsive or steric interactions, is modeled by the EFP in the quantum hamiltonian. The directed electrostatics and distributed polarizabilities included in the EFP provide the local effective dielectric for the active site. Repulsive interaction-EFPs have been generated for a variety of protein residues [19] by fitting the repulsive interaction over a range of interactions found for the hydrogen-bonded conformations in the active site. All EFP are obtained at the Hartree-Fock level so that the calculation of the entire EFP and all-electron system is essentially a Hartree-Fock calculation for a very large system that can include much of the protein environment.

All geometry optimizations and transition state searches are done at the restricted Hartree-Fock level in GAMESS [31] using the effective core potential (ECP) to restrict the all-electron calculation to the valence electrons [38,39]. Since the number of all-electron atoms is relatively large, requiring a substantial basis set (~ 300-700) for the representation of the wavefunction, the calculation is restricted to a CEP 4-31G basis concomitant to the ECP. Polarized basis sets have not had a substantial effect on the structure of a single metal active site [40,41]. Structural calculations have been compared with a DFT calculation for phosphotriesterase with reasonable agreement for the native structures including zinc substitution by cadmium [16]. Using the EFP to analyze metal-substitution and protonation of the active site in phosphotriesterase has already proved accurate [20].

ACTIVE SITE STRUCTURE AND SUBSTRATE BINDING

All the structures for phosphotriesterase are leveraged from the Zn-Zn x-ray crystal structure [9]. The structure for the Cd-Cd enzyme is not used to initiate any calculations. The Cd-Cd active site structure is calculated from the Zn-Zn and shown to agree well with experiment for metal-ligand distances as well as angles within the first-shell [20]. This provides one test of the efficacy of the EFP for the determination of the structure of the active site. Although the metal-metal distance changes by over 0.4 Å from Zn-Zn to Cd-Cd, the increase is predominately obtained by distortion in the first-shell. The crystal structures show that the backbone atoms of the Zn and Cd enzymes superimpose within 0.2 Å. The differences in the *in vacuo* and EFP structures are found primarily in the orientation of the ligands that are constrained by the hydrogen-bonds to the interior residues. All metal substituted enzymes can be leveraged from the Zn-Zn using the EFP protein environment.

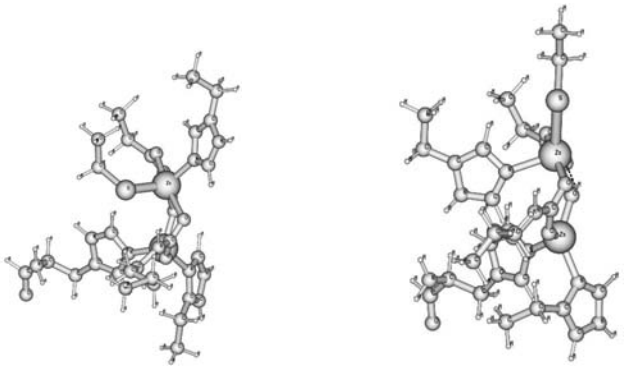
The active site of phosphotriesterase is described in fig.1 with the protein environment directly influencing the first-shell. The first-shell consists of the two metal cations and the following ligands: D301, H55, H57, H230, H201, K169 with the lysine carbamylated. All these residues are considered all-electron including all atoms back to the C_α atom. In one case, H230, the amide backbone is included since there are backbone hydrogen-bonded interactions to the interior protein. The residues represented by the by EFP are W131, D232, D233, D253, H254, H257, L271, M317, F306, and Y309. If all the atoms represented in the EFP calculation were represented by all-electron basis functions, there would be a total of 1244 basis functions rather than the 538 required. For the substrate binding calculations, W131, will also be considered all-electron and optimized since it can interact with the bound substrate. In the case of a paraoxon-like substrate, H257 would also be all-electron. H254 is chosen to be protonated because the arrangement of hydrogen-bonds suggests this is probable. H257 is found to be important in the chiral selection [14] but it probably functions through hydrogen bonding to the nitro group in the substituted paraoxons since such a hydrogen bond is easily modeled when the phosphonate inhibitor is converted into paraxon. In phosphotriesterase, the interior or protein has hydrophilic and hydrogen bonding interactions, which modify the charge distribution in the first shell by

polarization and charge transfer, while the exterior side toward solution determines how the protein shapes the hydrophobic box into which the substrate binds. Disruption of the pattern of hydrogen-bonds into the interior of the protein is the basis for structural and specificity changes that occur when mutations are made in the second and third shell around the active site [7].

HXC MUTANT STRUCTURE

The structures of all histidine to cysteine mutations in the first-shell of the active site in phosphotriesterase are optimized with the Cá frozen for all binding ligands. Although, the cysteine sulfur initially in the optimization can be as much as 4-5 Å from the zinc in the first shell, the active-site complex distorts sufficiently to permit ligand binding with a Zn-S distance of about 2.3-2.8 Å. Ligand binding of the cysteine was obtained with all four mutants with both bridging ligands maintained in the optimized structure. In an unconstrained optimization, the carbamylated lysine bond to Zn1 is strained and essentially broken for the H55C and H57C mutants. However, the strain in moving the Cá cannot be assessed at this time. The H55 and H57 structures only are shown in fig.2. Relevant bond distances for the active site are provided in Table 1.

The relative activities of all the HXC mutants for paraoxon have been determined [15]. The H55C mutant shows no activity while H57C activity increased over the native with a smaller reduction in the other two mutants. There are no obvious structural effects of the mutations that would affect activity. The relative effective charges at Zn1 and Zn2, however, do produce a surprise. In the native Zn-Zn enzyme, the Lowdin effective charge is comparable between Zn1 and Zn2. However, Zn1 is more positive than Zn2 for H57C, H201C, and H230C but it is less positive for H55C. The effective charges support the contention that Zn2 does not play a catalytic role in binding to the phosphoryl oxygen. The reactive conformation of paraoxon in the mutants will probably resemble the inhibitor structure in the Zn-Zn structure with no direct phosphoryl binding to the metal cations.



H55C H57C

Figure 2. HXC mutant structure showing just the first-shell for a. H55, b. H57.

TABLE 1. Selected active site bond distances for HXC (Å).

	H55C	H57C	H201C	H230C
Zn-Zn	3.58	3.66	3.45	3.48
Zn1-Oasp	2.09	2.03	2.11	2.00
Zn1-OH	1.91	2.21	1.98	2.05
Zn1-Olys	2.47	2.84	5.71	5.20
Zn1-S	2.47	2.84	5.71	5.20
Zn2-OH	1.91	1.90	1.98	1.97
Zn2-Olys	1.97	1.99	2.07	1.98
Zn2-S	5.13	6.41	2.37	2.38

H257 and W131 are EFP in the present calculations. They play no role in the mutant structure but shifts in their positions could have an inordinate effect on paraoxon binding so they would have to be all-electron in any further calculation on the reaction path. The reactive conformations with bound waters are required to obtain insight into reactive behavior. Unconstrained optimizations also suggest that the carbamylated lysine bridge to Zn1 can be broken. This carboxyl anion can then assist the activation of a water for nucleophilic attack. The mutant geometrical and electronic structure is insufficient by itself to provide insight into the activity.

BINDING OF WATER AND TRIMETHYLPHOSPHATE

Calculation on the active site structure of phosphotriesterase finds ligated water or a water hydrogen-bonded to the first-shell active site ionic ligands is very polarized. In addition to the effects this has on the structure, we propose that this polarized water can act as the nucleophile. By including water hydrogen-bonded to the active site ionic center, this provides more room for docking the substrate without having to eject the water. Such a structure has been calculated for phosphotriesterase with a trimethylphosphate substrate. Trimethylphosphate was chosen as perhaps the simplest organophosphate and it is easy to generalize to other models where the methyl group is replaced by fluorine or the phosphate oxygen replaced by a sulfur. Trimethylphosphate probably reacts very slowly since there is no good leaving group. The triethyl phosphate is found bound to the active site but not directly to the metal [11]. Apparently any substituent larger than the methyl group will sterically interact with the tryptophan or phenyalanine that border the Zn2 site. Of course, a halogen substituent will fit easily. Two structures are found with water hydrogen bonded to the bridging hydroxide (3a) or the carbamylated lysine (3b). The O(P) distance to the Zn1 or Zn2 is about 2.2 Å while triethyl phosphate is observed to bind about 3.4 Å from the Zn2 or more solvent accessible site [11].

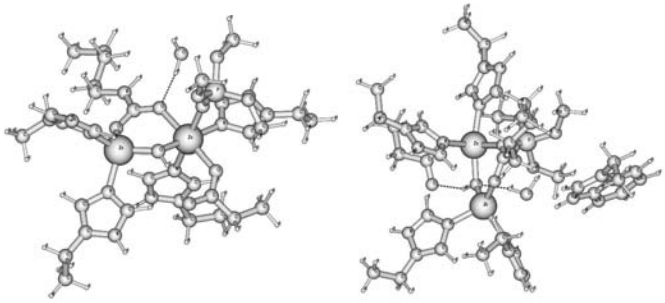


Figure 3. Binding of trimethylphosphate and water to phosphotriesterase, a. Water bound to carbamylated lysine, b. Water bound to bridging hydroxide.

The activation energy for hydrolysis of this relatively unreactive reactant is calculated at about 20 kcal/mol.The transition state is late and product-like as suggested by experiment. The methanol product is obtained by an intra-molecular proton transfer from the OH(P) to the methoxy oxygen which is comparable to the gas-phase attack of trimethylphosphate by hydroxide [42].

CONCLUSIONS

Theoretical calculations show that a single crystal structure of the Zn-Zn phosphotriesterase can be leveraged to determine the active site structure of the metal-substituted enzyme (Cd-Cd), low pH or protonated form of the active site, and mutants of active site residues (HXC). This provides a means of producing a wide range of active site structures relevant to the reaction or binding of interesting substrates by theoretical calculation. A new reaction pathway is also suggested by the calculations and critical analysis of the experimental crystal structures where the hydrolysis is initiated with an polarized water that is hydrogen-bonded to the active site ionic residues. Electronic structure of the native and mutant active sites suggest that a substrate with small substituents can bind equally well at either Zn1 or Zn2 sites. Binding of the simple organophosphate, trimethylphosphate, in the active site supports this contention. More complicated substrates will not bind at the metal sites but are suspended over the metals in the active site with the phosphate within easy distance of attack by a water hydrogen-bonded in the active site. The hydroxide will remain bridging but the carbamylated lysine bridge may open and be involved in the reaction.

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